

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 31/00, 31/56		A1	(11) International Publication Number: WO 97/07789 (43) International Publication Date: 6 March 1997 (06.03.97)
(21) International Application Number: PCT/GB96/02134		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 28 August 1996 (28.08.96)			
(30) Priority Data: 9517622.8 29 August 1995 (29.08.95) GB			
(71) Applicant (for all designated States except US): THE UNIVERSITY OF EDINBURGH [GB/GB]; Old College, South Bridge, Edinburgh EH8 9YL (GB).			
(72) Inventors; and			
(75) Inventors/Applicants (for US only): WALKER, Brian, Robert [GB/GB]; University of Edinburgh, Dept. of Medicine, Western General Hospital, Edinburgh EH4 2XU (GB). EDWARDS, Christopher, Richard, Watkin [GB/GB]; Imperial College School of Medicine, Imperial College, Level 5 Sherfield Building, London SW7 2AZ (GB). SECKL, Jonathan, Robert [GB/GB]; University of Edinburgh, Molecular Medicine Centre, Western General Hospital, Edinburgh EH4 2XU (GB).			
(74) Agent: MANATON, Ross, Timothy; J.Y & G.W. Johnson, Kingsbourne House, 229-231 High Holborn, London WC1V 7DP (GB).			

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: REGULATION OF INTRACELLULAR GLUCOCORTICOID CONCENTRATIONS

(57) Abstract

The interconversion of inactive 11-keto steroids with their active 11β -hydroxy equivalents can be controlled by the use of inhibitors of the 11β -reductase enzyme, such as carbenoxolone (3β -(3-carboxypropionyloxy)-11-oxo-olean-2-en30-oic acid). Such inhibitors may be put to a number of therapeutic uses in humans and animals, for instance to inhibit hepatic gluconeogenesis, to lower intracellular cortisol concentration, to increase insulin sensitivity in adipose tissue and muscle, and to prevent or reduce neuronal loss/cognitive impairment due to glucocorticoid potentiated neurotoxicity or neural dysfunction or damage.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

REGULATION OF INTRACELLULAR GLUCOCORTICOID CONCENTRATIONS

This invention relates to the interconversion of inactive 11-keto steroids with their active 11 β -hydroxy equivalents, to methods by which the conversion of the 5 inactive to the active form may be controlled, and to useful therapeutic effects which may be obtained as a result of such control. More specifically, but not exclusively, the invention is concerned with interconversion between cortisone and cortisol in humans.

10 Glucocorticoids such as cortisol have a number of diverse effects on different body tissues. For example, the use of cortisol as an anti-inflammatory agent was described in our International Patent Application WO 90/04399, which was concerned with the problem that therapeutically 15 administered cortisol tends to be converted in the body to inactive cortisone by 11 β -hydroxysteroid dehydrogenase enzymes. Our earlier invention provided for the potentiation of cortisol by the administration of an inhibitor of the 11 β -dehydrogenase activity of these enzymes.

20 Another major physiological effect of cortisol is its antagonism to insulin, and it is known for example that high concentrations of cortisol in the liver substantially reduce insulin sensitivity in that organ, thus tending to increase gluconeogenesis and consequently raising blood sugar levels 25 [1]. This effect is particularly disadvantageous in patients suffering from impaired glucose tolerance or diabetes mellitus, in whom the action of cortisol can serve to exacerbate insulin resistance. Indeed, in Cushing's syndrome, which is caused by excessive circulating 30 concentrations of cortisol, the antagonism of insulin can provoke diabetes mellitus in susceptible individuals [2].

As mentioned above, it is known that cortisol can be converted in the body to cortisone by the 11 β -dehydrogenase activity of 11 β -hydroxysteroid dehydrogenase enzymes. It is 35 also known that the reverse reaction, converting inactive

- 2 -

cortisone to active cortisol, is accomplished in certain organs by 11β -reductase activity of these enzymes. This activity is also known as corticosteroid 11β -reductase, cortisone 11β -reductase, or corticosteroid 5 11β -oxidoreductase.

It has only recently become apparent that there are at least two distinct isozymes of 11β -hydroxysteroid dehydrogenase (collectively abbreviated as 11β -HSD, which term is used, where appropriate, in this specification).
10 Aldosterone target organs and placenta express a high affinity NAD⁺-dependent enzyme (11β -HSD2) [3]. This has been characterised in placenta and kidney [4,5] and cDNA clones have been isolated [6,9]. 11β -HSD2 catalyses 11β -dehydrogenase activity exclusively [4,7]. In contrast,
15 the previously purified, liver derived isozyme (11β -HSD1) is a lower affinity, NADP⁺/NADPH-dependent enzyme [10,11]. Expression of 11β -HSD1 in a range of cell lines encodes either a bi-directional enzyme [11,12] or a predominant
20 11β -reductase [13,15] which, far from inactivating glucocorticoids, regenerates active 11β -hydroxysteroid from otherwise inert 11-keto steroid . 11β -reductase activity, best observed in intact cells, activates 11-keto steroid to alter target gene transcription and differentiated cell function [13,14]. 11β -HSD1 and 11β -HSD2 are the products of
25 different genes and share only 20 % amino acid homology [6,7].

As far as the applicants are aware no previous attempts have been made to modify the action of 11β -reductase. We have now found that it is possible to inhibit this activity
30 *in vivo*, and in doing so we have created the possibility of a novel medicament for use in treating many of the deleterious effects of glucocorticoid excess. In one aspect, therefore, the invention provides the use of an inhibitor of 11β -reductase in the manufacture of a medicament for control
35 of 11-keto steroid conversion to 11β -hydroxysteroid *in vivo*.

As mentioned above, one of the major physiological

- 3 -

effects of cortisol is insulin antagonism in the liver, and in a specific aspect the invention therefore provides the use of an inhibitor of 11β -reductase in the manufacture of a medicament for inhibiting hepatic gluconeogenesis [1].
5 Cortisol promotes hepatic gluconeogenesis by several mechanisms, including antagonism of the effects of insulin on glucose transport, and interactions with insulin and glucose in the regulation of several enzymes which control glycolysis and gluconeogenesis. These include glucokinase,
10 6-phosphofructokinase, pyruvate kinase, phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase. Inhibiting production of cortisol from cortisone in the liver therefore enhances hepatic glucose uptake and inhibits hepatic glucose production by several mechanisms [16]. Moreover, the
15 influence of inhibiting 11β -reductase activity in the liver of patients with insulin resistance or glucose intolerance may be greater than in healthy subjects because in insulin resistance or deficiency the influence of cortisol on PEPCK has been shown to be greater [17]; obese patients secrete
20 more cortisol [18]; insulin resistant patients are more sensitive to glucocorticoids [19]; and insulin down-regulates 11β -HSD1 expression [15,20] so that 11β -reductase activity may be enhanced in conditions of insulin resistance or deficiency.

25 Our studies have also shown that 11β -HSD1 is expressed in rat adipose tissue and in adipocyte cell lines in culture, where it converts 11-dehydrocorticosterone to corticosterone (the rat equivalents of human cortisone and cortisol, respectively). This suggests that similar
30 11β -reductase activity will be observed in human adipose tissue, with the result that inhibition of the enzyme will result in alleviation of the effects of insulin resistance in adipose tissue in humans. This would lead to greater tissue utilisation of glucose and fatty acids, thus reducing
35 circulating levels. The invention therefore provides, in a further aspect, the use of an inhibitor of 11β -reductase in the manufacture of a medicament for increasing insulin sensitivity in adipose tissue.

- 4 -

The results of our studies have encouraged us to believe that inhibition of intracellular cortisol production will also lead to increased insulin sensitivity in other tissues which are acted upon by insulin, for instance 5 skeletal muscle [21]. Inhibiting the 11β -reductase therefore promises to reverse the effects of insulin resistance in muscle tissue, and to promote the up-take of essential molecules such as glucose and free fatty acids into muscle cells with consequent improved muscle metabolism and 10 reduction of circulating levels of glucose and fatty acids. In a further aspect, the invention therefore provides the use of an inhibitor of 11β -reductase in the manufacture of a medicament for increasing insulin sensitivity in skeletal muscle tissue.

15 It is also known that glucocorticoid excess potentiates the action of certain neurotoxins, which leads to neuronal dysfunction and loss. We have studied the interconversion between $11\text{-dehydrocorticosterone}$ and corticosterone in rat hippocampal cultures, and have found (surprisingly in view 20 of the damaging effects of glucocorticoids) that 11β -reductase activity dominates over 11β -dehydrogenase activity in intact hippocampal cells [22]. The reason for this activity is unknown, but this result indicates that glucocorticoid excess may be controlled in hippocampal cells 25 (and by extension in the nervous system in general) by use of an 11β -reductase inhibitor, and the invention therefore provides in an alternative aspect the use of an inhibitor of 11β -reductase in the manufacture of a medicament for the prevention or reduction of neuronal dysfunction and loss due 30 to glucocorticoid potentiated neurotoxicity. It is also possible that glucocorticoids are involved in the cognitive impairment of ageing with or without neuronal loss and also in dendritic attenuation [23-25]. Furthermore, glucocorticoids have been implicated in the neurological 35 dysfunction of major depression. Thus an inhibitor of 11β -reductase could also be of value in these conditions.

It will be appreciated from the foregoing that the

- 5 -

potential beneficial effects of inhibitors of 11β -reductase are many and diverse, and it is envisaged that in many cases a combined activity will be demonstrated, tending to relieve the effects of endogenous glucocorticoids in diabetes mellitus, obesity (including centripetal obesity), neuronal loss and the cognitive impairment of old age. Thus, in a further aspect, the invention provides the use of an inhibitor of 11β -reductase in the manufacture of a medicament for producing multiple therapeutic effects in a patient to whom the medicament is administered, said therapeutic effects including an inhibition of hepatic gluconeogenesis, an increase in insulin sensitivity in adipose tissue and muscle, and the prevention of or reduction in neuronal loss/cognitive impairment due to glucocorticoid-potentiated neurotoxicity or neural dysfunction or damage.

From an alternative point of view, the invention provides a method of treatment of a human or animal patient suffering from a condition selected from the group consisting of: hepatic insulin resistance, adipose tissue insulin resistance, muscle insulin resistance, neuronal loss or dysfunction due to glucocorticoid potentiated neurotoxicity, and any combination of the aforementioned conditions, the method comprising the step of administering to said patient a medicament comprising a pharmaceutically active amount of an inhibitor of 11β -reductase.

As mentioned previously, the factors which control the relative activities of 11β -dehydrogenase and 11β -reductase in different conditions, especially by the 11β -HSD1 isozyme, are poorly understood. It is likely that an 11β -reductase inhibitor will be selective for the 11β -HSD1 isozyme *in vivo*. We have found, for instance, that glycyrrhetic acid (a known inhibitor of 11β -dehydrogenase) has no effect on 11β -reductase *in vivo* [26]. However, we have surprisingly found that carbenoxolone, which is known as an inhibitor of the 11β -dehydrogenase enzyme, also inhibits 11β -reductase *in vivo* [26, 27]. In preferred embodiments, therefore, the

- 6 -

inhibitor is carbenoxolone (3β -(3-carboxypropionyloxy)-11-oxo-olean-2-en 30-oic acid), or a pharmaceutically acceptable salt thereof. The dose of carbenoxolone which we used in our studies was 100 mg every 5-8 hours given orally.

The invention is hereinafter described in more detail by way of example only, with reference to the following experimental procedures and results and the accompanying figures, in which:-

10 Figure 1 is a graph showing dextrose infusion rates (M values) after placebo or carbenoxolone administration, in an euglycaemic hyperinsulinaemic clamp study carried out on humans;

15 Figure 2 is a graph showing fasting plasma glucose concentrations in rats after placebo or carbenoxolone therapy at the daily doses indicated;

Figure 3 is a graph illustrating the effect of estradiol administration to gonadectomised or gonadectomised/adrenalectomised rats on hepatic 11β -HSD1 activity, and mRNA expression for 11β -HSD1 and PEPCK.

Figure 4 contains graphs of data obtained from mice which are either homozygous for the wild type 11β -HSD1 allele or which are homozygous for a knockout mutant allele for 11β -HSD1;

25 Figure 5 illustrates 11β -HSD1 enzyme activities and mRNA expression in undifferentiated and differentiated 3T3-F442A cells;

Figure 6 is a graph illustrating the effect of pretreatment with corticosterone and 11-dehydrocorticosterone with or without carbenoxolone, upon rat hippocampal cell survival on exposure to kainic acid.

- 7 -

EXPERIMENTAL RESULTS IN SUPPORT OF THE INVENTION

A. INSULIN SENSITIVITY

A.1 EFFECT OF CARBENOXOLONE ON INSULIN SENSITIVITY IN MAN

Consistent with the observations in animal tissues and cultured cells described above, we have established the relative activities of 11β -dehydrogenase and 11β -reductase *in vivo* in different organs in man by measuring the cortisol/cortisone ratio in plasma from selective venous catheterisations [28]. In most organs the venous effluent contains cortisol and cortisone in a ratio of ~10:1. However, in renal vein plasma the ratio is 3:1, while in hepatic vein plasma the ratio is 55:1, consistent with potent 11β -dehydrogenase activity due to 11β -HSD2 expression in the kidney and potent 11β -reductase 11β -HSD1 activity in the liver. Moreover, cortisone taken orally, and therefore delivered to the liver via the portal vein, is avidly converted on first pass to cortisol in the peripheral circulation [26,27]. Using this index of 11β -reductase activity we have shown that, in addition to inhibiting renal 11β -dehydrogenase activity, carbenoxolone (but not glycyrhetic acid) [26,27] inhibits hepatic 11β -reductase activity *in vivo* in man.

In the liver, mineralocorticoid receptors are not expressed in significant numbers, but glucocorticoid receptors are abundant. Surprisingly, the affinity of glucocorticoid receptors for cortisol is 10-40 times lower than that of mineralocorticoid receptors [29]. It may be that, by contrast with the protection of high-affinity mineralocorticoid receptors from cortisol required of 11β -dehydrogenase activity in the distal nephron, 11β -reductase activity in the liver is required to ensure that low-affinity glucocorticoid receptors get adequate exposure to cortisol. The free circulating concentrations of cortisol and cortisone are approximately equal, so that a large pool of cortisone is available for activation in the

- 8 -

liver. Such a mechanism would be analogous to the intra-cellular activation of other members of the thyroid-steroid hormone family which circulate in relatively inactive forms [30], such as testosterone which is converted 5 by 5 α -reductase to dihydrotestosterone, and thyroxine which is converted by 5'-monodeiodinase to tri-iodothyronine.

To test this hypothesis, we have used carbenoxolone to inhibit hepatic 11 β -reductase, and observed changes in hepatic glucocorticoid receptor activation inferred 10 indirectly from changes in insulin sensitivity. Hepatic insulin sensitivity decreases and hepatic glucose production increases during both pharmacological [31, 32] and physiological [33] increases in glucocorticoid levels. The results of these experiments were published [16] after the 15 filing of our British patent application number 9517622.8 on 29 August 1995.

Methods

Seven non-obese (Body Mass Index <25) healthy male Caucasian volunteers on no medication, aged 27-36 years, 20 were given carbenoxolone (100 mg every 8 hours orally) or matched placebo for seven days in a randomised double-blind cross-over design, with phases separated by at least 4 weeks. On the seventh day of each phase euglycaemic hyperinsulinaemic clamp studies with measurement of forearm 25 glucose uptake were performed, as described in detail in reference 16, the content of which is incorporated herein by reference.

Results

Carbenoxolone administration was associated with no 30 change in fasting glucose concentration but a fall in fasting insulin concentration. During the euglycaemic clamp, carbenoxolone enhanced the metabolic clearance rate of glucose (M values: $41.1 \pm 2.4 \mu\text{b}5\text{mol/kg/min}$ for placebo versus 44.6 ± 2.3 for carbenoxolone; $p<0.03$) (Figure 1). No 35 difference was detected in peripheral glucose uptake as measured by forearm glucose uptake, but this measurement was

- 9 -

less precise due to insulin-induced vasodilatation [34] and the mental stress induced by the restraint of the study conditions.

Discussion

5 These data show that carbenoxolone increases whole-body insulin sensitivity. This could have resulted from either increased suppression of hepatic glucose production as a reflection of increased hepatic insulin sensitivity, or increased peripheral utilisation of glucose as a reflection
10 of increased peripheral insulin sensitivity. The absence of a change in forearm glucose uptake with carbenoxolone suggests that the former mechanism predominates. However, the imprecision of this measurement raises the possibility that there is a contribution from enhanced peripheral
15 insulin sensitivity which we have not detected. A more detailed discussion of these data has now been published [16].

It is unlikely that carbenoxolone affects insulin sensitivity by a mechanism independent of its effect on
20 11 β -reductase. Carbenoxolone inhibits other enzymes which metabolise cortisol, notably 5 β -reductase [35], but this effect would increase intra-hepatic cortisol concentrations and reduce insulin sensitivity. In the absence of corticosteroids, carbenoxolone at this dose has no
25 documented effects *in vivo* and low affinity for corticosteroids receptors *in vitro* [36], so that a direct action of carbenoxolone to increase insulin sensitivity is unlikely. Indeed, previous experiments suggest that, at higher concentrations (mmol/l), carbenoxolone without
30 corticosteroid antagonises the action of insulin in adipocytes [37]. Blood pressure and forearm blood flow were not elevated by carbenoxolone in this study, and even if they were this might be associated with reduced insulin sensitivity [34]. Finally, other neurohumoral changes
35 consequent on the renal effects of carbenoxolone (suppressed plasma renin, aldosterone, and potassium concentrations) are not known to influence insulin sensitivity directly, and

- 10 -

in the context of oral sodium loading were associated with decreased insulin sensitivity [38].

From these observations we infer that basal 11 β -reductase activity plays a role in maintaining adequate 5 exposure of glucocorticoid receptors to cortisol in human liver. The circulating pool of cortisone is therefore physiologically important as a source of active glucocorticoid in sites where 11 β -reductase is expressed.

A.2 EFFECT OF CARBENOXOLONE ON INSULIN SENSITIVITY IN THE 10 RAT

Following our studies of the effects of carbenoxolone in healthy humans, we have performed unpublished studies of the effects of carbenoxolone in intact healthy rats. Consistent with the observations in human, we have shown 15 that rat hepatocytes in primary culture express 11 β -HSD1 which has predominant 11 β -reductase activity [15]. We have also shown that the circulating concentration of 11-dehydrocorticosterone (measured by gas chromatography and mass spectrometry) is 40-50 nmol/l (Dr R Best, unpublished 20 observation) so that there is a substantial pool of inactive 11-ketosteroid available for activation to corticosterone in sites where 11 β -reductase is active.

Methods

Intact male Wistar Hans rats of body weight 200-250 g 25 were treated with daily subcutaneous injection of 1 ml of either 0.9% saline (vehicle) or carbenoxolone (made up to 1, 3, or 10 mg/ml in 0.9% saline) for 14 days. They were fasted from 4 pm and blood obtained by tail-nick at 9 am the next day. Plasma glucose was measured by a glucose oxidase method 30 on a Beckman CX-3 autoanalyser.

Results

Carbenoxolone produced a dose-dependent fall in fasting glucose concentrations (Figure 2).

- 11 -

Discussion

These experiments are preliminary, since it is now our intention to assess insulin sensitivity and glucose tolerance in rats treated with carbenoxolone. However, the fall in fasting plasma glucose is consistent with inhibition of hepatic gluconeogenesis with or without enhanced peripheral glucose uptake in rats treated with the 11β -reductase inhibitor carbenoxolone.

A.3 EFFECT OF ESTRADIOL-MEDIATED INHIBITION OF HEPATIC 10 11β -HSD1 EXPRESSION ON INSULIN SENSITIVITY IN THE RAT

Having established the effects of a competitive pharmacological inhibitor of 11β -reductase activity on insulin sensitivity and glucose levels in man and rat, the object of this study was to examine the effects of 15 down-regulation of transcription and translation of the 11β -HSD1 enzyme. The results of these studies have been presented at scientific meetings during 1996 but are not yet published.

11β -HSD1 expression is regulated by a variety of 20 hormones *in vivo* [26]. In the rat liver, 11β -HSD1 shows pronounced sexual dimorphism, with lower activity in females. Indeed, chronic estradiol administration almost completely suppresses hepatic 11β -HSD1 expression in both male and female rats [39-41]. This regulation appears to be 25 tissue- and isozyme-specific, as estrogen does not attenuate 11β -HSD1 expression in the hippocampus or 11β -HSD2 activity in the kidney [41]. The aim of this study was therefore to examine the contribution which reactivation of glucocorticoids by hepatic 11β -HSD1 makes to the expression 30 of liver-specific glucocorticoid modulated genes in the rat by exploiting the selective suppression of hepatic 11β -HSD1 by estradiol *in vivo*.

Methods

In-vivo studies

35 Male Han Wistar rats (200-250 g) underwent gonadectomy

- 12 -

and either bilateral adrenalectomy or sham-operation under halothane anaesthesia. For estradiol administration, silicone elastomer capsules (1.95 mm internal diameter, 3.125 mm external diameter) (Dow Corning Corporation, 5 Midland, Michigan, USA) containing 17β -estradiol (Sigma, Poole, UK) were implanted subcutaneously. For animals treated for 42 days with estradiol, the capsules were removed and replaced after 21 days. Control animals were implanted with blank capsules. Adrenalectomised rats were 10 maintained on 0.9% saline. Rats were killed 10, 21 or 42 days after surgery. Liver and hippocampus were removed and dissected on ice for assay of 11β -HSD activity and an aliquot of liver was frozen on dry ice and stored at -80°C until extraction of RNA.

15 *Quantitation of 11β -HSD activity in in vivo experiments*

Tissues were homogenised in Krebs-Ringer bicarbonate buffer with 0.2% glucose, pH 7.4, and assayed as described previously [40]. Homogenates (6.25 µg of liver protein, 250 µg of hippocampal protein) were incubated with 200µM NADP 20 (Sigma, Poole, UK) and 12 nM [3 H]-corticosterone (specific activity 84 Ci/mmol; Amersham International, Aylesbury, UK) in a total volume of 250 µl with Krebs-Ringer buffer supplemented with 0.2% bovine serum albumin for 10 min at 37°C. 11β -dehydrogenase activity was quantified in this 25 assay as a measure of active enzyme since 11β -reductase is unstable in homogenates. Steroids were extracted with ethyl acetate and separated by HPLC with on-line β counter. 11β -HSD activity was expressed as conversion of corticosterone to 11 -dehydrocorticosterone, after correction 30 for apparent conversion in incubates without enzyme (<3%).

Extraction and analysis of mRNA

Total RNA was extracted from liver tissue by the guanidinium thiocyanate method, as described [40] and resuspended in diethylpyrocarbonate-treated water. RNA 35 concentration and purity was assayed spectrophotometrically. 20 µg aliquots were separated on a 1 .2% agarose gel containing 2% formaldehyde. RNA was blotted onto

- 13 -

nitrocellulose membranes (Hybond-N, Amersham International, UK), prehybridized in 6 ml phosphate buffer (0.2 M NaH₂PO₄, 0.6 M NaH₂PO₄, 5 mM EDTA), 3 ml 20% SDS and 100 µg denatured herring testis DNA (Sigma, Poole, UK) for 2 h at 55°C and 5 hybridized at 55°C overnight in the same solution containing rat 11β-HSD1 or PEPCK cDNA, labelled with ³²P-dCTP using a random primed DNA labelling kit (Boehringer Mannheim UK Ltd., Lewes, UK). Three 20 min washes were carried out at room temperature in 1xSSC (0.3 M NaCl, 0.03 M sodium citrate), 0.1% SDS followed by a stringent wash at 55°C for 30 min in 0.3xSSC, 0.1% SDS. Filters were exposed to autoradiographic film for 1-4 days (adjusted to ensure the signal density was within the linear range). Filters were rehybridised with similarly labelled 7S cDNA or transferrin 15 RNA cDNA probes to control for loading, as previously described [40] (transferrin mRNA expression is not altered by estradiol or glucocorticoid). Optical density was determined using a computer-driven image analysis system (Seescan plc, Cambs, UK). Values were expressed as a 20 percentage of control levels.

Statistics

Data are the means±SEM of 5-10 replicates (indicated in figure legends). Data were compared by ANOVA and Newman-Keuls post-hoc test or by Student's unpaired t-test, 25 as appropriate. Statistical tests were carried out on absolute data, but the figures display data as percent of values for control animals to improve clarity.

Results

Effect of chronic estradiol treatment on 11β-HSD1 30 activity and mRNA expression

Estradiol administration for 10, 21 and 42 days to gonadectomised male rats resulted in marked decreases in hepatic 11β-HSD activity (Figure 3). However, 11β -HSD activity was not completely abolished since prolonged (60 35 min) incubation of liver homogenates from rats given estradiol for 42 days showed detectable (12.2±14.7%)

- 14 -

conversion of corticosterone to 11-dehydrocorticosterone. 11 β -HSD1 mRNA expression fell to undetectable levels after 21 and 42 days of estradiol treatment. 11 β -HSD activity in the hippocampus was not altered by estradiol treatment at 21 or 42 days (data not shown).

Effect of estradiol on hepatic glucocorticoid-inducible gene expression

To examine the effect of attenuated hepatic 11 β -HSD1 activity on local glucocorticoid action, the expression of 10 liver-specific glucocorticoid-inducible genes was measured after 42 days of estradiol. PEPCK mRNA expression was significantly reduced by estradiol treatment (Figure 3).

To examine whether these effects of estradiol on hepatic gene expression were mediated directly or via 15 alterations in glucocorticoid action in the liver, the effects of estradiol in adrenalectomised animals or sham-operated controls was examined. Animals were killed after 21 days. Plasma corticosterone values confirmed the adequacy of adrenalectomy (data not shown). Adrenalectomy 20 increased hepatic 11 β -HSD1 gene expression and activity compared with sham-operated controls (Figure 3). Estradiol reduced liver 11 β -HSD1 mRNA and activity in adrenalectomised rats, and although mRNA expression was higher than estradiol-treated adrenally-intact rats, it remained 25 significantly reduced compared with untreated controls.

Hepatic PEPCK gene expression was decreased after 21 days of estradiol treatment, albeit to a slightly lesser extent than after 42 days (Figure 3). Unsurprisingly, adrenalectomy also attenuated hepatic PEPCK gene expression, 30 but in adrenalectomised rats estradiol no longer reduced, but indeed increased PEPCK mRNA levels when compared with adrenalectomy alone.

- 15 -

Discussion

Estradiol administration for both 21 and 42 days produced undetectable hepatic 11β -HSD1 mRNA expression and markedly reduced 11β -HSD activity (although this remained clearly measurable), confirming previous studies [41]. The discrepancy between mRNA expression and activity might be due to much slower turnover of 11β -HSD1 protein or, more likely, to the transcription and translation of residual low levels of 11β -HSD1 mRNA, undetectable by northern analysis.

This effect of sex steroids is relatively specific to the liver, since hippocampal 11β -HSD1 was unaffected, and previous studies have shown that 11β -HSD2 is not attenuated by estrogens [22]. Thus the advantage of exploiting estrogen down-regulation of hepatic 11β -HSD1, as opposed to conventional carbenoxolone or other inhibitors of the enzyme, is that 11β -HSD2 is unaffected.

Following 42 days of estradiol treatment, hepatic expression of mRNA encoding PEPCK was clearly reduced. In principle, this effect of chronic estrogen treatment may be explained in several ways: (i) estrogen may act directly to repress hepatic gene expression; (ii) estradiol may alter corticosteroid metabolism in the liver. Reduced hepatic 11β -HSD1-mediated reactivation of otherwise inert 11-dehydrocorticosterone is anticipated to reduce expression of these glucocorticoid-sensitive transcripts; (iii) there may be other indirect effects of chronic estradiol administration.

For PEPCK direct inhibition by estradiol appears unlikely, since this transcript was induced by estrogen in adrenalectomised rats. The loss of the attenuating effect of estradiol on PEPCK expression in adrenalectomised rats suggests a requirement for glucocorticoids in the process, rather than any other indirect effect. Glucocorticoids increase expression of PEPCK in the liver [17] and so this pattern of changes is compatible with the glucocorticoid control of expression of this gene. The effect of estradiol in intact rats was as potent as adrenalectomy alone in

- 16 -

attenuating PEPCK mRNA expression. These data are therefore consistent with the effects of estradiol being indirectly mediated via the marked loss of 11 β -HSD1 11 β -reductase activity, which thus attenuates intrahepatic glucocorticoid regeneration. Thus the 11 β -reductase activity of 11 β -HSD1 is likely to be of key importance in producing sufficiently high intrahepatic corticosterone levels to elevate PEPCK above the minimum levels maintained by basal (non-glucocorticoid) factors.

10 A.4 EFFECT OF TRANSGENIC KNOCKOUT OF 11 β -HSD1 EXPRESSION

Although the effects of carbenoxolone and estrogen-induced downregulation of 11 β -HSD1 expression provide strong support for our hypothesis that 11 β -reductase inhibition will enhance insulin sensitivity and reduce hepatic glucose production, both of these agents are potentially confounded by other actions, including direct effects on 11 β -HSD2 (carbenoxolone) and other effects on the liver (estrogen). The most clear-cut evidence of the importance of a specific protein can now be tested most effectively using transgenic technology. We have now produced a transgenic mouse which is homozygous for a mutant allele carrying a targetted disruption of the 11 β -HSD1 gene. Some of these results have been presented at scientific meetings during 1996 but have not yet been published.

25 Methods

A mouse genomic library constructed from isogenic OLA129 DNA in the lambda vector GEM12 was screened with a rat 11 β -HSD1 cDNA probe. A lambda phage containing a 14 kb insert encompassing exons 2 to 5 of the mouse 11 β -HSD1 gene was cloned and subjected to extensive restriction mapping and sequence analysis. Using this information, a replacement vector 16 Kpn β A was constructed using the pBS-KpnA cassette. The vector consisted of the neomycin resistance gene under the control of the human β -actin promoter and followed by the SV40 polyadenylation signal. The 6.5 kb long 3' homology arm was subcloned from intron D. The 1.2 kb short homology

- 17 -

arm was synthesised by PCR using high fidelity Ultma Taq polymerase, 129 mouse genomic DNA and primers bearing nested Sac I and Spe I restriction sites and located in exon 1 and intron B. An external probe was synthesised by PCR. Using this probe, a specific recombination event generates a 1.5 kb *BamHI* fragment instead of a 3 kb fragment expected for the wild type gene.

Following transfection of the 16 *Kpnβ A* replacement construct into 'CGR 8' embryonic stem cells, of 368 10 neomycin-resistant colonies screened, only one showed homologous recombination at the 5' end. The specificity of the recombination event was tested by Southern blot hybridisation after *BamHI* restriction. Specificity of restriction at the 3' end was also confirmed by 15 hybridisation with an internal probe after three different restrictions.

Positive embryonic stem cells were injected into C57Bl/6 blastocysts to generate chimeric offspring who were crossbred to establish a colony of homozygotes for the 20 knockout allele.

Results

Homozygotes for the mutant allele are morphologically intact and fully fertile. No survival bias is conferred by the mutant allele. However, neither 11 β -HSD1 mRNA nor enzyme 25 activity (techniques as above) were detected in mutant homozygotes, and reduced mRNA and activity were detected in heterozygotes. Plasma corticosterone levels are not altered in mutants, but the ratio of corticosterone to 11-dehydrocorticosterone in urine is markedly reduced. 30 Homozygous mutant mice are also unable to convert 11-dehydrocorticosterone to corticosterone *in vivo* and are thus resistant to the thymic involution seen with 11-dehydrocorticosterone in wild-type control mice.

Animals were fasted for 48 hours before sacrifice and 35 collection of trunk blood. Plasma glucose was measured by a

- 18 -

glucose oxidase method (see above). Fasting plasma glucose concentrations were lower in mutant animals (Figure 4).

Measurement of enzymes responsible for hepatic glycolysis/gluconeogenesis revealed no differences between 5 wild type and mutant animals in the fed state. However, on fasting the mutant animals failed to show the normal induction of glucose-6-phosphatase and PEPCK (Figure 4). Glucokinase was not altered.

Discussion

10 These data demonstrate the importance of 11β -HSD1 in hepatic glucose metabolism and insulin sensitivity. Glucose-6-phosphatase and PEPCK are two gluconeogenic enzymes which are down-regulated by insulin and up-regulated by glucocorticoid exposure [1]. In the presence of insulin 15 (in fed animals) there is no change in expression of these enzymes, confirming that insulin regulation is dominant. However, in starved animals with low insulin levels there is a failure of induction of these enzymes consistent with a failure of corticosterone-dependent induction. In animals in 20 which the mutant vector has recombined specifically to knockout only 11β -HSD1, and in which plasma corticosterone is maintained by normal hypothalamic-pituitary-adrenal feedback these changes can be attributed to impaired intra-hepatic conversion of 11-dehydrocorticosterone to 25 corticosterone.

A.5 ROLE OF 11β -HSD1 IN ADIPOCYTE MATURATION

Glucocorticoids are involved in triggering adipocyte differentiation from uncommitted adipoblasts into committed preadipocytes. Glucocorticoid excess in humans causes 30 profound changes in both adipose tissue distribution and metabolism [18]. In Cushing's syndrome, omental fat shows adipose cell hyperplasia, associated with enhanced lipoprotein lipase and glycero-phosphate dehydrogenase activity. In homogenised adipose tissue cortisol and 35 corticosterone are oxidised to cortisone and

- 19 -

11-dehydrocorticosterone, indicating the presence of 11β -HSD [42] which might modify glucocorticoid action in a site- and developmentally-specific manner. We have (i) examined the expression of 11β -HSD enzyme activity and 11β -HS1 mRNA in 5 isolated primary rat adipocytes; and (ii) determined whether 11β -HSD1 is expressed in a fibroblast derived clonal cell line, 3T3-F442A which can be induced into mature adipocytes in presence of fetal calf serum and insulin. The results of this work have not been published.

10 Methods

Animals

Epididymal adipose tissue was excised from adult male Wistar rats. Adipose tissue was washed several times in Phosphate Buffered Saline, trimmed of large blood vessels 15 and minced. Tissue was incubated in Krebs-Ringer buffer (KRB) containing collagenase II (2 mg/ml) (Sigma, UK) at 37°C for 40 min, and the digested material passed through a 250 μ m nylon filter and briefly centrifuged. The separated adipocyte (floating) and stromal vascular (pellet) fractions 20 were collected for (i) RNA extraction and northern blot as described above and (ii) 11β -dehydrogenase bioactivity assay as described above, using final concentrations of 500 μ g protein/ml, 200 μ M cofactor (NADP or NAD), and 12 nM 1,2,6,7-³H-corticosterone in a total volume of 250 μ l 25 Krebs-Ringer buffer for 15 min at 37°C.

Cell culture

Clonal preadipocyte cell lines 3T3-F442A, (kindly provided by Dr. Pairault Henri Modor Hospital, Creteil, France) were plated at a density of 10^4 cells/100 mm diameter 30 dishes in 'basal medium' (Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% newborn calf serum, penicillin 200 U/ml and streptomycin 50 μ g/ml). To differentiate confluent 3T3-F442A cells, basal medium was replaced with 'differentiation medium' (DMEM, 10% fetal calf serum and 35 insulin, 5 μ g/ml) and maintained in this for 11 days with the medium changed every 48 h.

- 20 -

11 β -Dehydrogenase and 11 β -reductase activities were measured in intact cells by addition of 25 nM corticosterone with labelled ^3H -corticosterone or 25 nM 11-dehydrocorticosterone with labelled ^3H -11-dehydrocorticosterone. Interconversion of corticosterone and 11-dehydrocorticosterone was assessed as above at 3, 8 and 24 h after the addition of steroids.

Results

Extracts from homogenized rat adipocytes showed 11 β -HSD activity, as demonstrated by conversion of corticosterone to 11-dehydrocorticosterone. 11 β -HSD1 was transcribed in whole white adipose tissue and isolated adipocytes as a single, approximately 1.6kb species of mRNA, of similar size to the hepatic species, though expression was lower than in liver. 11 β -HSD1 transcripts were also expressed in the stromal vascular fraction (data not shown).

3T3-F442A cells were induced to differentiate and, after 10 days, >90% cells were differentiated as determined by visible lipid accumulation. Associated with this differentiation there was a marked increase in 11 β -HSD1 mRNA expression and 11 β -reductase activity which occurred late (8 days after addition of differentiation medium) and in association with induction of GPDH expression. (Figure 5).

Discussion

In this study, we have demonstrated 11 β -HSD1 gene expression in rat adipose tissue and isolated rat adipocytes, as well as the stromal vascular preadipocyte fraction, in agreement with data demonstrating 11 β -HSD activity in the adipose component of the mammary gland [42]. By contrast with data obtained in homogenised tissue and in keeping with findings in other whole cell preparations [15, 22], we have found in whole cells that 11 β -HSD1 operates as an 11 β -reductase rather than 11 β -dehydrogenase enzyme. In addition, in the 3T3-F442A cell line, we have demonstrated that 11 β -HSD1 expression is regulated in a differentiation-dependent manner. 11 β -HSD1 thus has the

- 21 -

characteristics of a 'late' differentiation gene. Moreover, the regulated expression of 11β -HSD1 paralleled that of GPDH, a glucocorticoid-sensitive late marker of adipocyte differentiation.

5 These results support the hypothesis that 11β -reductase plays a physiological role in converting 11-keto steroids to 11β -hydroxy steroids and thus amplifying glucocorticoid activity. In the adipocyte this may explain the induction of GPDH. Although inhibition of 11β -reductase would not be
10 expected to influence the early differentiation of adipocytes, it may influence the biochemical phenotype of differentiated cells, specifically in relation to lipid metabolism.

B NEURONAL EFFECTS OF 11β -REDUCTASE INHIBITORS

15 Several studies have demonstrated 11β -HSD activity, immunoreactivity and mRNA expression in hippocampal neurons [43] 45. Administration of 11β -HSD inhibitors alters functional activity in the hippocampus *in vivo* [46], although the mechanisms underpinning this effect are
20 obscure.

Previous studies have shown that in homogenates of hippocampus, both dehydrogenation and reduction occur [44], but the reaction direction in intact cells was previously unknown. We have now examined 11β -HSD activity and its
25 function in primary cultures of fetal hippocampus cells. The results of these experiments were published [22] after the filing of our patent application number 9517622.8 on 29 August 1995. Further details of the experiments will be found in reference 22, the contents of which are
30 incorporated herein by way of reference.

Methods and Results

Hippocampi were dissected from Wistar rat embryos on day 18.5 of pregnancy and cultured in DMEM with supplements, as described [22]. These cells expressed 11β -HSD1 mRNA, and

- 22 -

homogenised cells performed either 11β -dehydrogenase or 11β -reductase activity. However, in whole cells in culture, only 11β -reductase activity was demonstrated. This 11β -reductase activity could be almost abolished by the 5 addition of carbenoxolone (10^{-6} M) [22].

To assess the influence of 11β -reductase activity on neurotoxicity, cells were exposed to kainic acid and cell survival determined. Both corticosterone and 10 11 -dehydrocorticosterone potentiated kainic acid neurotoxicity. However, carbenoxolone did not alter the effect of corticosterone, but protected the cells from the potentiating effect of 11 -dehydrocorticosterone (Figure 6).

Discussion

These data demonstrate that 11β -HSD1 is an 15 11β -reductase enzyme in rat hippocampus and that the conversion of 11 -ketosteroids to 11 -hydroxysteroids potentiates the neurotoxic action of glucocorticoids. Importantly, they show that inhibition of 11β -reductase activity can protect the cells from the damage that occurs 20 due to reactivation of 11 -dehydrocorticosterone. This supports the hypothesis that inhibition of 11β -reductase in human brain would prevent reactivation of cortisone into cortisol and protect against deleterious glucocorticoid-mediated effects on neuronal survival and 25 other aspects of neuronal function, including cognitive impairment, depression, and increased appetite.

REFERENCES

1. Dallman MF, Strack AM, Akana SF, et al. *Front Neuroendocrinol* 14, 303-347 (1993).
- 30 2. Ross EJ & Linch DC. *Lancet* 2, 646-649 (1982).
3. Walker BR, Campbell JC, Williams CRW. *Endocrinology* 131, 970-972 (1992).
4. Brown RW, Chapman KE, Edwards CRW & Seckl JR *Endocrinol* 132, 2614-2621 (1993).
- 35 5. Rusvai E & Náray-Fejes-Tóth A J *Biol Chem* 268, 10717-

- 23 -

- 10720 (1993).
6. Agarwal AK, Mune T, Monder C & White PC (1994) J Biol Chem 269, 25959-25962 (1994).
 7. Albiston AL, Obeyesekere VR, Smith RE & Krozowski ZS
5 Mol Cell Endocrinol 105, R11-R17 (1994).
 8. Náray-Fejes-Tóth A & Fejes-Tóth G. Endocrinology 136, 2579-2586 (1995).
 9. Brown RW, Chapman KE, Koteletsev Y, et al. Biochem J 313, 1007-1017 (1996).
 10. Lakshmi V & Monder C Endocrinol 123, 2390-2398 (1988).
 11. Agarwal AK, Monder C, Eckstein B & White PC J Biol Chem 264, 18939-18943 (1989).
 12. Agarwal AK, Tusie-Luna M-T, Monder C & White PC Mol Endocrinol 4, 1827-1832 (1990).
 13. Duperrex H, Kenouch S, Gaeggleler HP, et al. Endocrinology 132, 612-619 (1993).
 14. Low SC, Chapman KE, Edwards CRW & Seckl JR Journal of Molecular Endocrinology 13, 167-174 (1994).
 15. Jamieson PM, Chapman KE, Edwards CRW & Seckl JR.
20 Endocrinology 136, 4754-4761 (1995).
 16. Walker BR, Connacher AA, Lindsay RM, Webb DJ & Edwards CRW. J Clin Endocrinol Metab 80, 3155-3159 (1995).
 17. Friedman JE, Yun JS, Patel YM, McGrane MM & Hanson RW. J. Biol Chem 268, 12952-12957 (1993).
 18. Bjorntorp P. J Intern Med 230, 195-201 (1991).
 19. Walker BR, Seckl JR & Phillips DIW. J. Endocrinol 148(suppl), P370 (1996). (Abstract)
 20. Hammami MM & Siiteri PK. J Clin Endocrinol Metab 73, 326-334 (1991).
 21. Monder C & Shackleton CHL. Steroids 44, 383-417 (1984).
 22. Rajan V, Edwards CRW & Seckl JR. J Neurosci 16, 65-70 (1996).
 23. Landfield PW, Waymire J & Lynch G Science 202, 1098-1102 (1978).
 24. Sapolsky RM, Krey LC & McEwen BS J Neurosci 5, 1221-1226 (1985).
 25. Sapolsky RM, Krey LC & McEwen BS Endocr Rev 7, 284-301 (1986).
 26. Walker BR, Edwards CRW. Endocrinol Metab Clin N Amer.

- 24 -

- 23(2), 359-377 (1994).
27. Stewart PM, Wallace AM, Atherden SM, Shearing CH, Edwards CRW. Clin Sci. 78, 49-54 (1990).
28. Walker BR, Campbell JC, Fraser R, Stewart PM, Edwards CRW. Clin Endocrinol (Oxf). 97, 483-492 (1992).
- 5 29. Arriza JL, Weinberger C, Cerelli G. Science 237, 268-275 (1987).
30. Stewart PM & Sheppard MC. Mol. Cell Endocrinol. 83, C13-18 (1992).
- 10 31. de Feo P, Perriello G, Torlone E, et al. Am J Physiol. 257, E35-E42 (1989).
32. Rooney DP, Neely RDG, Cullen C, Ennis CN, Sheridan B, Atkinson AB. J Clin Endocrinol Metab. 101, 1180-1183 (1994).
- 15 33. Dinneen S, Alzaid A, Miles J, Rizza R. J. Clin Invest. 92, 2283-2290 (1993).
34. Tappy L, Randin D, Vollenweider P, et al. J Clin Endocrinol Metab. 101, 1063-1069 (1994).
35. Latif SA, Conca TJ, Morris DJ. Steroids. 55, 52-58
20 (1990).
36. Armanini D, Karbowiak I, Funder JW. Clin Endocrinol (Oxf). 79, 609-612 (1983).
37. Gomez-Capilla JA, Gutierrez C, Fernandez-Fernandez JM. Biochem Pharmacol. 37, 1299-1301 (1988).
- 25 38. Donovan DS, Solomon CG, Seely EW, Williams GH, Simonson DC. Am J Physiol. 264, E730-E734 (1993).
39. Lax ER, Ghraf R & Schriefers H. Acta Endocrinol (Copenh) 89, 352-358 (1978).
40. Low SC, Moisan M-P, Edwards CRW & Seckl JR. J
30 Neuroendocrinol 6, 285-290 (1994).
41. Low SC, Chapman KE, Edwards CRW Wells T, Robinson ICAF & Seckl JR. J Endocrinol 143, 541-548 (1994).
42. Quirk SJ, Slattery J & Funder JW. J Steroid Biochem 35, 623-625 (1990).
- 35 43. Moisan M-P, Seckl JR & Edwards CRW Endocrinol 127, 1450-1455 (1990).
44. Lakshmi V, Sakai RR, McEwen BS & Monder C. Endocrinol 128, 1741-1748 (1991).
45. Sakai RR, Lakshmi V, Monder C & McEwen BS. J

- 25 -

Neuroendocrinol 4, 101-106 (1992).

46. Seckl JR, Dow RC, Low SC, Edwards CRW & Fink G. J Endocrinol 136, 471-477 (1993).

- 26 -

CLAIMS

1. The use of an inhibitor of 11β -reductase in the manufacture of a medicament for the control of 11-keto steroid conversion to $11\beta\text{-hydroxysteroid}$ in vivo.

5 2. The use according to claim 1, for the control of cortisone conversion into cortisol in humans.

3. The use according to claim 2, for lowering hepatic cortisol concentration.

4. The use according to claim 3, for inhibiting 10 hepatic gluconeogenesis.

5. The use according to claim 2, for lowering intracellular cortisol concentration.

6. The use according to claim 5, for increasing insulin sensitivity in adipose tissue.

15 7. The use according to claim 5, for increasing insulin sensitivity in muscle.

8. The use according to claim 5, for the prevention or reduction of neuronal dysfunction or loss/cognitive impairment due to glucocorticoid potentiated neurotoxicity 20 or neural dysfunction or damage.

9. The use of an inhibitor of 11β -reductase in the manufacture of a medicament for producing multiple therapeutic effects in a patient to whom the medicament is administered, said therapeutic effects including an 25 inhibition of hepatic gluconeogenesis, an increase in insulin sensitivity in adipose tissue and muscle, and the prevention of or a reduction in neuronal dysfunction, damage or loss due to glucocorticoid potentiated neurotoxicity.

- 27 -

10. The use according to any preceding claim, for the treatment of diabetes mellitus, impaired glucose tolerance, or glucocorticoid associated cognitive or affective disorder.

5 11. The use according to any preceding claim, in which the 11β -reductase inhibitor is carbenoxolone (3β -(3-carboxypropionyloxy)- 11 -oxo-olean-2-en 30-oic acid), or a pharmaceutically acceptable salt thereof.

12. A method of treatment of a human or animal patient
10 suffering from a condition selected from the group consisting of: hepatic insulin resistance, adipose tissue insulin resistance, muscle tissue insulin resistance, neuronal loss due to glucocorticoid potentiated neurotoxicity, and any combination of the aforementioned
15 conditions, the method comprising the step of administering to said patient a medicament comprising a pharmaceutically active amount of an inhibitor of 11β -reductase.

13. A method according to claim 12, wherein said inhibitor is selected from the group consisting of
20 carbenoxolone (3β -(3-carboxypropionyloxy)- 11 -oxo-olean-2-en 30-oic acid), and pharmaceutically acceptable salts of carbenoxolone.

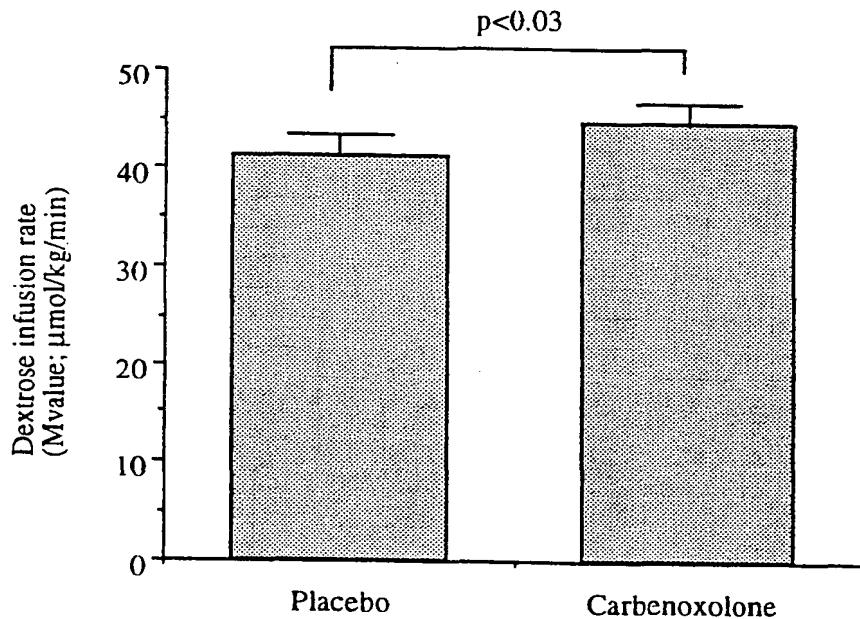
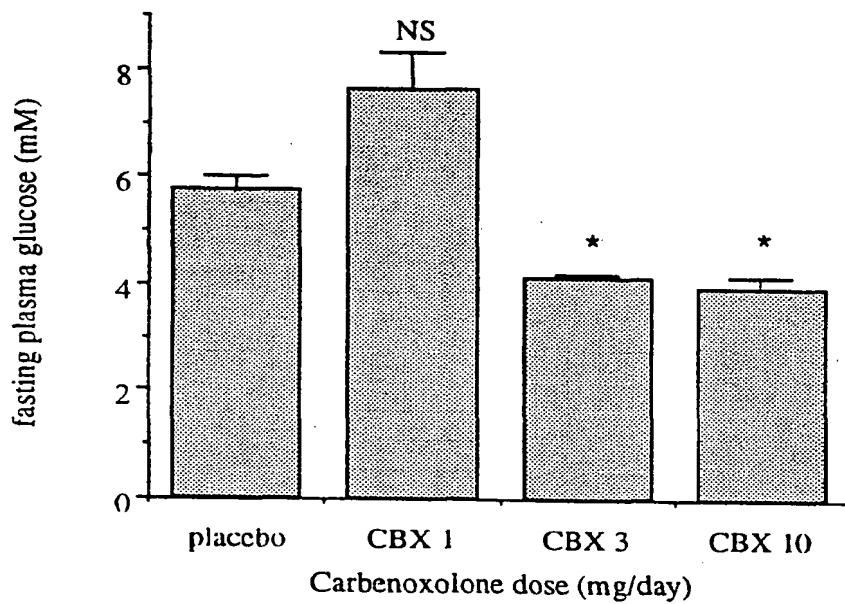


Figure 2



ANOVA: $p<0.001$

* indicate $p<0.05$ compared with placebo

NS = not significant

2/3

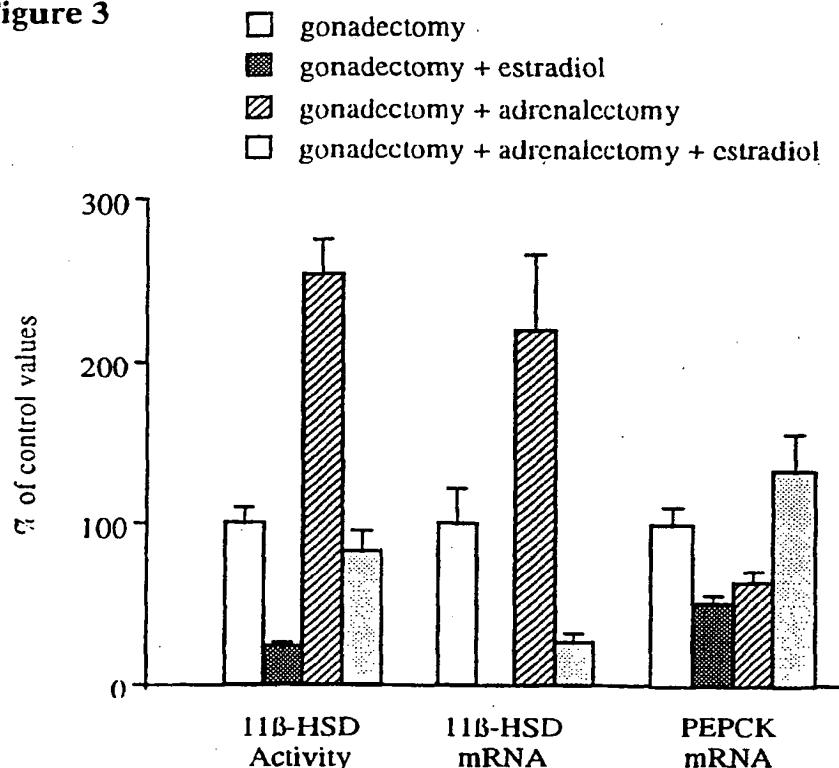
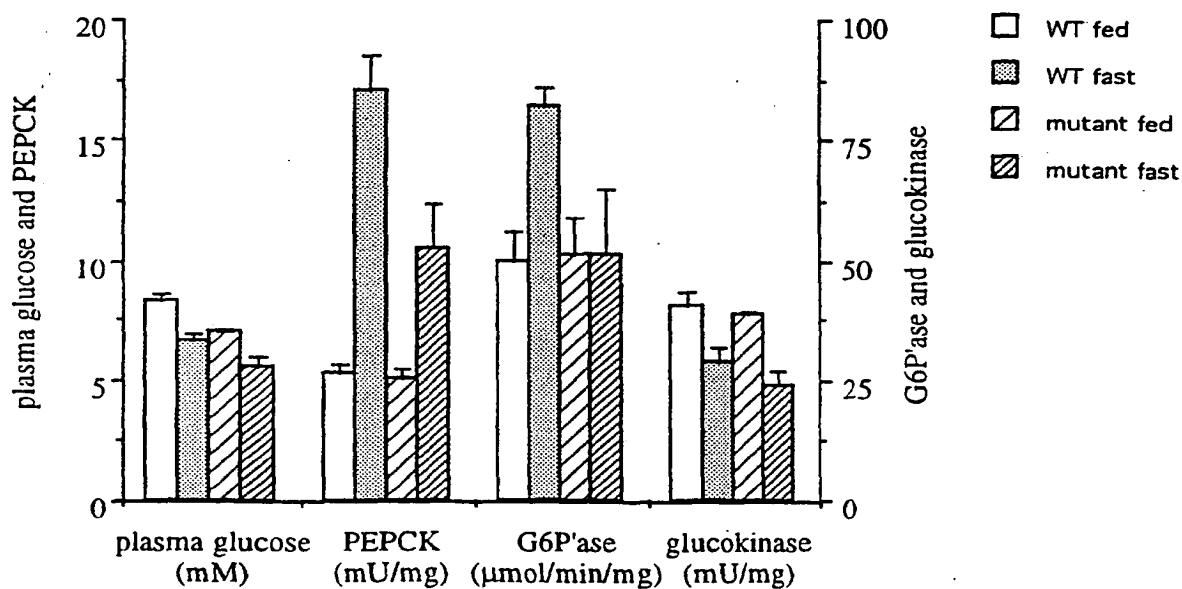
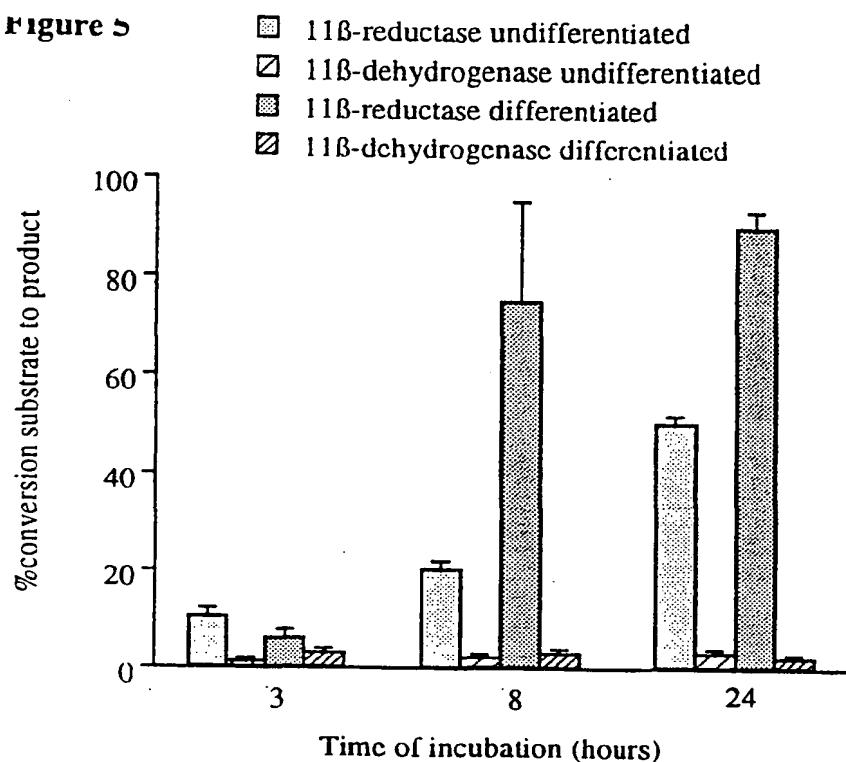
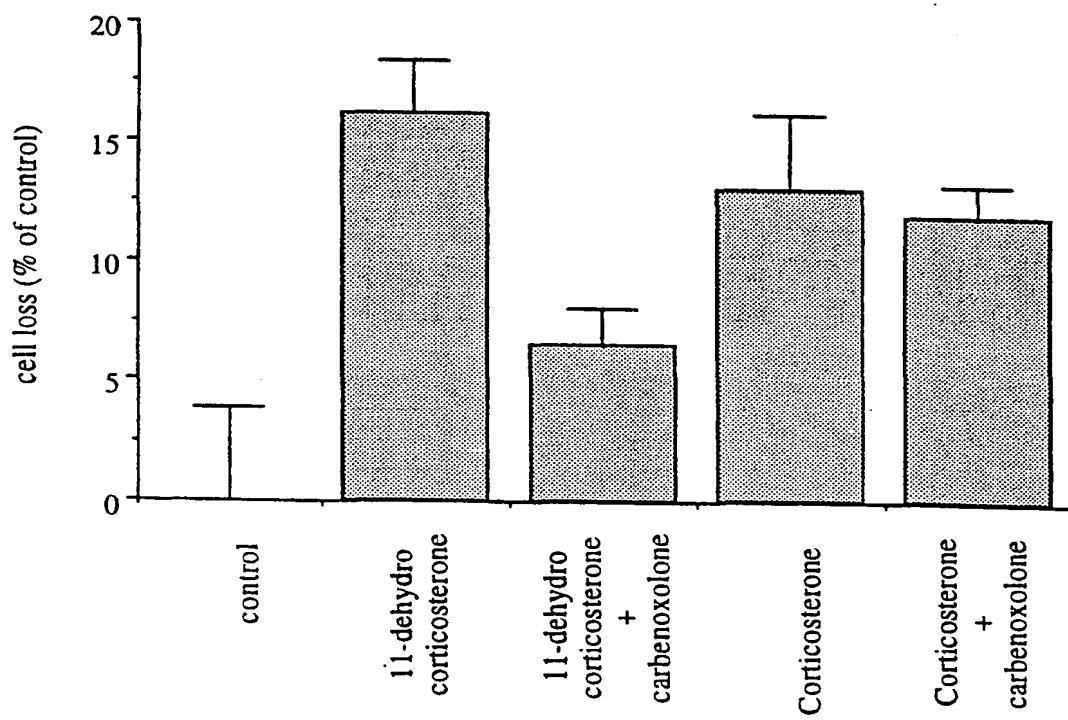
Figure 3**Figure 4**

Figure 5**Figure 6**

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/02134

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 A61K31/00 A61K31/56		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. STEROID BIOCHEM. MOL. BIOL., vol. 40, no. 4-6, 1990, pages 501-509, XP000613038 P.M. STEWART ET AL: "The cortisol-cortisone shuttle and hypertension." see page 506 ---	1,2,11
X	CLIN. ENDOCRINOL., vol. 39, no. 2, 1993, pages 221-227, XP000612640 B.R. WALKER ET AL.: "Deficient inactivation of cortisol by 11 beta-hydroxysteroid dehydrogenase in essential hypertension." see page 224 ---	1,2,11 -/-
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input type="checkbox"/> Patent family members are listed in annex.		
<p>* Special categories of cited documents :</p> <ul style="list-style-type: none"> *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed 		
<p style="text-align: center;">*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p style="text-align: center;">*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p style="text-align: center;">*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p style="text-align: center;">*&* document member of the same patent family</p>		
Date of the actual completion of the international search		Date of mailing of the international search report
18 December 1996		17.01.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016		Authorized officer Klaver, T

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/02134

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>J. CLIN. ENDOCRINOL. METAB., vol. 80, no. 11, 1995, pages 3155-3159, XP000612666</p> <p>B.R. WALKER ET AL.: "Carbenoxolone increases hepatic insulin sensitivity in man: a novel role for 11-oxosteroid reductase in enhancing glucocorticoid receptor activation." see the whole document</p> <p>-----</p>	1-5,9

1